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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

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JAN 2 9 1974

APPLICATION FOR RESEARCH GRANT

Submitted: 1/15/73
Revised: 1/16/74

1. CO-PRINCIPAL INVESTIGATORS

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3. DEPARTMENTS WHERE RESEARCH WILL BE DONE OR COLLABORATION PROVIDED:

Pulmonary Disease Section and
Research Laboratories
Albert Einstein Medical Center

4. SHORT TITLE OF STUDY: LUNG PROTEINASE: ANTIPROTEINASE BALANCE AND THE EFFECT OF CIGARETTE SMOKE ON THIS INTERACTION

5. PROPOSED STARTING DATE: May 1, 1974.

6. ESTIMATED TIME TO COMPLETE: 3 years.

7. BRIEF DESCRIPTION OF SPECIFIC RESEARCH AIMS:

The twin objectives of this investigation are 1) to identify and quantitate the primary factor responsible for protecting the lung against the action of autogenous proteinases which have been previously shown in this laboratory to produce experimental emphysema in dogs - and 2) to examine the role of cigarette smoke on this interaction. We shall isolate, purify, characterize and quantitate the normally occurring substance, found in lung tissue which inhibits the activity of specific proteolytic enzymes and determine if this lung antiproteinase found in dogs has its counterpart in the human lung. The production of antiproteinases and their ability to interact with enzymes capable of inducing experimental emphysema in animals will be studied and evaluated

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in normal animals and those exposed to cigarette smoke. In order to bring this study as close as possible to the human condition, we will use human material (i.e. polymorphonuclear leukocytes, alveolar macrophages and lungs) which will be available to us by collaboration with the Medical Examiners' Office, the Division of Laboratories at Albert Einstein and Jeannes Hospital.

8. BRIEF STATEMENT OF WORKING HYPOTHESIS:

Our working hypothesis is that emphysema may be induced by the proteolytic activity of specific enzymes from polymorphonuclear leukocytes and/or pulmonary macrophages. The release of these enzymes may be stimulated by various air-borne pollutants. These enzymes overwhelm the local defense mechanisms in the lung, including such factors as serum and tissue antiproteinases, and destroy or alter the elastic tissue of the alveoli.

9. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES:

PART I - INTRODUCTORY STATEMENT

Epidemiologic studies suggest that smoking is an important factor in the development of pulmonary emphysema in humans. Two major, non-mutually exclusive theories for its induction differ only in their emphases on initial tissue attack. In the vascular theory (1), the initiating lesion is of vascular origin, resulting in obstruction of branches of the bronchial or pulmonary blood supply. The subsequent deficiency of nutrients would then lead to necrosis of alveolar walls and septa to form the characteristic emphysema pathology. According to the second theory (2), emphysema develops due to the direct attack of proteolytic enzymes at the air-lung interface. Regardless of which theory is correct there is eventual extensive damage to both the vascular and parenchymal tissue. A logical source of this degradation could conceivably be intracellular collagenase and/or elastase, released into the surrounding tissues due to cellular necrosis caused by such stimuli as chronic infection or chronic pollution-induced destruction.

Although cigarette smoking and human emphysema have been statistically related using clinical and autopsy material, it has not yet been shown if there is a direct causal relationship or if smoking and other pollutants merely accelerate a normal aging process. Also, if there is a direct relationship between smoking and emphysema it is still possible that smoking is but one of a number of extrinsic factors able to act synergistically with autogenous agents to effect this lung damage. Those experimental studies which have been performed on the effects of smoking do, however, suggest reasons for exploring the action of cigarette smoke as a single contributing factor in emphysema induction.

When human pulmonary leukocytes from smokers and non-smokers were compared (3, 4, 5), even asymptomatic smokers had greatly increased numbers of primarily polymorphonuclears and macrophages with a direct correlation to the

amount of smoking and smoke inhaled. This cannot be explained as simply a protective effect, i.e., more phagocytes are supplied to remove an increased amount of foreign matter, because there was an actual decrease in the antibacterial activity of alveolar macrophages isolated from smokers. This effect differed among the various brands of cigarettes tested and varied inversely with the effectiveness of filtration (6). The significance of this decrease in antibacterial activity as opposed to such other findings as change in size or pigmentation, lies in the ability of the phagocytosed, undegraded particles to cause the lysis of the engulfing leukocyte with subsequent release of enzymes able to degrade pulmonary tissue.

The demonstrated greater susceptibility of cigarette smokers to respiratory infections may, therefore, be due to this combination of insufficient leukocyte degradation of invading organisms and the damage resulting from the action of liberated proteases on lung tissue (7). Since studies of human pulmonary emphysema are hampered by time required for induction and moral considerations, animal models have been employed. Although the horse has the advantage of having lung anatomy, distribution of bronchial arteries, and a natural emphysema similar to that found in humans, economy dictated that another animal model be used. Since this laboratory has successfully produced emphysema-like lesions in dogs (8), using aerosolized leukocyte proteinases and since the parenchymal effects of smoking in dogs resembled human emphysema (9), this system will be used as our initial model with the understanding that we will attempt to use human material when feasible in order to directly relate our observations to the human condition.

It has been suggested (10) that a decreased level of serum alpha$_1$-antitrypsin is of primary importance in correlating with the development of hereditary emphysema. Other investigators (7,11), however, have been unable to demonstrate a relationship between the development of emphysema and smoking in those individuals having intermediate or normal levels of this antiprotease. Although this may be a characteristic of the species studied, the report (12) that lung tissue binds the serum antiproteases suggests that not only is the total level of antiprotease important but also its localization and its availability to bind and thus inhibit proteolytic enzymes. Material from fractionated lung tissue must be examined for its role in proteinase-antiproteinase balance. Indeed, it is also possible that a lung specific antiproteinase may be present at the air-lung interface. Such antiproteinases may be of paramount importance as regulators of proteinases released from various cells in the lung during normal physiological states as well as during stress situations.

PART II - PREVIOUS WORK DONE ON THIS PROJECT

Since our original application was submitted we have much recent data in support of the concept of a lung antiproteinase acting as a primary defense system directed against those leukocyte enzymes capable of digesting lung tissue. Along these lines we have isolated, by lung lavage, an extracellular material which appears to have unique antiproteinase properties. The data are shown in Table I.

Table 1

Comparison of Various Antiproteinases
From Dog and Human Sources

Enzyme	Substrate	Maximum % Inhibition By: Dog Lung Inhibitor	Dog Serum	Human α_1-AT	Human α_2-MG	Human Lung Inhibitor
Purified Trypsin	BAPNA	22	87	70	14	--
	Casein	0	--	68	43	--
Pancreatic Elastase	t-BOC-Ala	91	50	95	30	0
	Casein	0	--	0	22	--
Dog PMN Elastase	t-BOC-Ala	93	70	100	22	--
Dog Alv. Macrophage Elastase	t-BOC-Ala	90	65	--	--	--
Human PMN Elastase	t-BOC-Ala	65	30	--	--	80

The data presented in Table I shows that dog lung inhibitor does not inhibit trypsin to any great extent (22%) whereas dog serum or human α_1-AT inhibit trypsin activity at least 70%. In addition, the lung antiproteinase is a more effective inhibitor of pancreatic elastase than the dog serum antiproteinases (91% vs. 50%). In this regard the dog lung inhibitor is markedly different than the human lung inhibitor, since the human lung antiproteinase does not appear to inhibit pancreatic elastase. In obtaining our human lung material from the Medical Examiners' Office we have arranged to get normal specimens (gunshot deaths, suicides or auto accidents) when the autopsy is performed within 2 to 3 hours of death. Collection of such material has been approved by our Committee on Research Involving Human Subjects.

The experiments described above show that the dog lung antiproteinase is different than the major dog or human serum antiproteinases. The data for human lung inhibitor was obtained from a 1M NaCl extract of human whole lung homogenate (Janoff, personal communication) and the material is contaminated with lung tissue proteins to a far greater extent than our saline lavage. We must now determine if saline lavage of human lung allows isolation of such a unique lung antiproteinase.

In addition, we have shown previously that polymorphonuclear leukocyte (PMN) homogenates will produce experimental emphysema in dogs and, more recently, have fractionated this PMN homogenate to isolate and characterize the emphysema-inducing agent. Acetone powders were prepared from dog PMN which were obtained by dextran sedimentation of fresh citrated dog blood. The desiccated material was extracted sequentially with water, 0.15M NaCl, 0.5M NaCl, 1.0M NaCl and 8M urea and the proteolytic activities of each of the fractions were determined using various substrates. General proteolysis against denatured hemoglobin was present in all the fractions. Elastolysis was found in the 1M NaCl and 8M urea fractions. Proteolysis using the residue remaining after 1M NaCl extraction of perfused, lavaged, dog lung homogenates as substrate was greatest in the 0.15M NaCl fraction, as is seen in Table II.

Table II

Characteristics of Various Dog PMN Fractions

PMN Fraction	Emphysema-Inducing Activity	Lung Residue Digestion Activity	% Inhibition of Lung Residue Digestion Using Lung Antiproteinase	Elastolytic Activity
H_2O	--	505	0	--
0.15M NaCl	++	1260	67	--
0.5M NaCl	+	924	64	--
1.0M NaCl	--	253	0	+
8.0M Urea	--	840	0	+

As can be seen in Table II, only the fractions capable of inducing experimental emphysema in excised dog lobes (0.15M and 0.5M) were inhibited by lung antiproteinase in their ability to digest lung tissue. The three other fractions were not inhibited by lung antiproteinase. Further purification of the 0.15M NaCl fraction by precipitation with 55% $(NH_4)_2SO_4$ and fractionation on CM-Sephadex increased markedly the purification of the emphysema-inducing activity. Emphysema production was inhibited by incubation of the active 0.15M NaCl fraction with ovomucoid, a known proteinase inhibitor. The PMN fraction responsible for emphysema production is a protease which is able to degrade lung tissue but cannot be described as having classically assayable collagenolytic or elastolytic properties (Table II).

Furthermore, we have partially purified and partially characterized the lung antiproteinase. The supernatant was concentrated 100-fold by either lyophilization or ultrafiltration yielding a solution containing approximately 5 mg/ml of protein. After dialysis against 0.01M phosphate buffer, pH 7.5, the sample was fractionated on DEAE cellulose using a discontinuous salt gradient. Stepwise elution with 0.03M, 0.06M, 0.10M and 0.50M NaCl in the phosphate buffer yielded four peaks of protein with a recovery of 85-100%. The distribution of protein was approximately 25% in peak I, 18% in peak II, 14% in peak III, 38% in peak IV. Most of the antiproteinase activity was recovered in peak II, measured by inhibition of hydrolysis of t-BOC-alanyl-p-NO_2 phenyl ester by porcine pancreatic elastase. Acrylamide gel electrophoresis at pH 7.0 in 1% sodium dodecyl sulfate showed marked enrichment of a high molecular weight component in peak II as compared to the other three peaks.

Immunologic techniques demonstrated that there were differences between this lung antiproteinase and serum antiproteinase. Antisera were prepared against whole dog serum as well as against unfractionated lung antiproteinase. Crude antiproteinase reacted with both antisera in Ouchterlony plates. Using immunoelectrophoresis the crude antiproteinase showed at least three arcs with anti-dog serum and at least two arcs

against antiserum to lung inhibitor. The partially purified antiproteinase of peak II showed a single arc against anti-dog serum. The arc was not coincident with that formed against partially purified dog alpha$_1$-antitrypsin.

This lung antiproteinase, while it showed immunologic cross-reactivity with serum proteins, was also differentiated from known dog serum antiproteinases on the basis of inhibition studies against two purified enzymes, trypsin and elastase. Under conditions where dog serum inhibited trypsin esterolysis maximally 90%, lung antiproteinase inhibited maximally 20%; whereas dog serum inhibited elastase esterolysis no more than 50% the lung antiproteinase inhibited more than 90%.

These data suggest that there may be a unique antiproteinase present at the air-lung interface. This inhibitor may play an important role in regulation of proteinases released into the lung under various physiological conditions.

With these data in mind, we propose the following research program.

PART III

A. RESEARCH PLAN OUTLINE

1. To Characterize Antiproteinase(s) in the Lung.

a. Isolate and characterize the factor(s) from dog and human lung responsible for antiproteinase activity.

b. Compare the factor(s) found in the dog and human lung with serum proteins demonstrating antiproteinase activity.

2. To Determine the Role of Smoking on the Interaction between the Proteinases and Antiproteinases in the Lung.

a. In vitro assay of proteinases from alveolar macrophages, lung tissue and PMN's and the qualitative and quantitative effects on them due to exposure to cigarette smoke.

b. In vitro assay of serum and lung antiproteinases from normal and smoking dogs.

c. Comparison of ease of induction of experimental emphysema using proteinases isolated from normal and smoking dogs on both types of dogs.

B. ABSTRACT OF RESEARCH APPROACH PROPOSED

1. To Characterize Antiproteinase(s) in the Lung

a. Isolate and characterize the factors from dog and human lung responsible for antiproteinase activity.

1) use at least two different fractionation systems (homotenization or lavage) to isolate dog and human lung antiproteinase

2) quantitate the amount of antiproteinase normally present in dog or human lung

3) purify the antiproteinase by chromatographic (DEAE, CM and Sephadex) procedures

4) determine its molecular weight, if there is sugar or lipid associated with the protein and the mechanism of inhibition of specific proteinases

5) determine if the lung antiproteinases can prevent the development of enzyme-induced emphysema in vivo in the dog, and in vitro in isolated human lung lobes

b. Compare the factor(s) found in the dog and human lung with serum proteins demonstrating antiproteinase activity.

1) analyze the purified lung and serum antiproteinases by acrylamide gel electrophoresis and peptide map characterization

2) determine if the antiproteinase of the lung is immunologically related to any of the serum antiproteinases

3) compare the inhibition spectra of specific enzymes by the lung and serum antiproteinases

2. To Determine the Role of Smoking on the Interaction Between the Proteinases and Antiproteinases in the Lung.

a. In vitro assay of proteinases from alveolar macrophages, lung tissue and PMN's and the qualitative and quantitative effects on them due to exposure to cigarette smoke.

1) assay fractionated proteinases from alveolar macrophages and PMN of normal subjects and those exposed to various regimens of cigarette smoke

2) assay release of proteinases from alveolar macrophages or PMN before and after exposure to cigarette smoke

b. The effect of smoking on serum and lung antiproteinase levels and activities.

1) assay sera and lung lavage for changes in antiproteinase activities comparing normal and smoking subjects

2) determine if there has been a change in the relative amounts of each type of serum inhibitor after smoking

3) establish if there is any immunologic change in any of the antiproteinases after smoking

c. Effect of cigarette smoke on induction of proteinase-induced.

1) establish if smoking makes the lung more susceptible to leukocyte-induced emphysema.

2) determine if the alveolar macrophages or PMN from smoking subjects are equally effective as normal cells in inducing emphysema.

C. EXPERIMENTAL PROCEDURE

1. Characterization of Lung Antiproteinases

Background

Human material will be studied not only as this is the species of primary concern, but also because no clearcut relationship has as yet been found between the intermediate levels of serum proteinase inhibitor and development of emphysema in smokers (11). One approach, that of genetic analysis of the numerous alleles involved in production of serum alpha$_1$-antitrypsin is being undertaken in several other laboratories (12). Our approach is a biochemical one in which we will evaluate the role of proteinase:antiproteinase balance under smoking and non-smoking conditions in relation to the development of experimental emphysema. Dog material will be employed due to the considerable body of data already available from this lab using this animal. Also, a direct relationship was found between macroscopic parenchymal disruption in dogs and duration of daily cigarette smoking over a period of 0-22 months (13). Microscopically, these lesions resembled human emphysema (13). Emphysema has been successfully produced in our laboratory in vivo using papain or leukocyte homogenate instillation or aerosolization. We have also developed an in vitro system for emphysema production utilizing isolated lobes and instillation of proteinases (14).

a. Isolation and Comparison of Dog and Human Lung Antiproteinases

Isolation and Quantitation of Tissue Inhibitors

Lung tissue from dog and human origin will be minced, homogenized in a blender, and washed three times with water followed by centrifugation at 15,000 rpm. The pellet will be washed twice, including once overnight, with 1M NaCl. The sodium chloride supernate will then be recentrifuged at 42,000 rpm (15). Supernate extracts will be assayed for their ability to inhibit the proteolytic activity of enzymes extracted from polymorphonuclear leukocytes using the hemoglobin assay (16). It is expected that antiproteinase activity will be found in this fraction because of the preliminary work of Lieberman (17) and Janoff (18). We will initiate this work using the NaCl extract, but will check other lung extraction procedures also. As mentioned in Part II we have already initiated an extraction procedure utilizing lung lavage and have found that this isolation procedure yields an antiproteinase which may be different than the Janoff inhibitor (see Table I). However, since Janoff's work was done with human lung and ours with dog lung we will use both procedures on both species of lung tissue in order to determine which technique will allow for easy isolation in a relatively pure form and permit quantitation of antiproteinase levels (mg/gm wet lung tissue; mg/mg surfactant phosphorus or mg inhibitor protein/dog).

Characterization of Inhibitors

In order to characterize these antiproteinases they will be purified using such standard procedures as precipitation with ammonium sulfate, methanol, trichloracetic or perchloric acid and various types of ion-exchange chromatography (19). An alternative procedure will be sought using the principle of affinity chromatography (20). Crystalline trypsin will be bound to Sepharose 4B using the cyanogen bromide method

of Cuatracasas and Anfinsen (21), and the matrix poured into a column. Lung extract or lavage containing the antiproteinase will then be applied to the column, nonbound protein washed off, and the adhering inhibitor selectively eluted. As was described in Part II, we have already developed a chromatographic procedure to partially purify lung inhibitor using DEAE cellulose columns. We will characterize the purified inhibitor as to molecular weight (determined by Sephadex and acrylamide gel electrophoresis in sodium dodecyl sulfate as well as by ultracentrifuge analysis). We will also determine if it is a glycoprotein or a lipoprotein and if it has any chemical or immunological relationship to surfactant, which is also present at the air-lung interface. Finally, we will establish more clearly the interaction between the antiproteinase and the enzymes listed in Tables I and II which it inhibits in order to understand the mechanism and requirements for inhibition.

Effect of Inhibitors on Induction of In Vitro Emphysema

In vitro emphysema has been induced in our laboratory using isolated lobes of dog lungs. Following removal of the lungs from the body the individual lobes were instilled with solutions of proteinase(s) and the enzyme(s) allowed to digest the tissues for 1½ hours at room temperature (1°). We will determine if the inhibitors isolated above will affect in vitro induction. Formaldehyde will be instilled into the lungs at a standard pressure of 25cm. The organs will be immersed in formaldehyde for 48 hours, mounted, sliced and stained. Sections will be examined for alveolar wall destruction using the method of Dunhill (22). This procedure will allow us to better define what we are considering to be emphysema-like lesions and also to quantitate the effects of various doses of agents required to produce a defined level of lung destruction. The use of isolated lobes will provide us with a considerable economic advantage over in vivo work employing the entire animal. It will also allow us to minimize the effect of any animal to animal variation on a given experiment. We have preliminary data showing that lung antiproteinase is a strong inhibitor of emphysema development in vitro and we will expand these studies to determine if this inhibition works in vivo. We will also attempt to induce emphysema-like lesions in human lobes in vitro using human PMN enzyme and establish if there is a human lung antiproteinase which can regulate disease induction in a manner similar to the dog system.

b. The Relationship between Lung and Serum Proteolytic Inhibitors

Although both the serum and the pulmonary tissues have been reported to contain substances able to inhibit proteolytic enzymes no one has studied the possible relationship between the inhibitors from these two sources. The value of such a comparison lies in the relationship of these inhibitors to both the etiology and prognosis of the disease state, always with the assumption that since proteolytic enzymes are significant in these processes so too are these inhibitors. If a serum inhibitor is actually a subunit of a lung inhibitor (or vice versa) then it could be expected that there would be some sort of quantitative relationship between their concentrations. Conditions regulating this ratio could

be involved in pathogenesis of pulmonary emphysema. Genetic abnormalities affecting the specific regulatory gene(s) would also be expected to affect both serum and pulmonary inhibitor if they were derived from the same molecule. Conversely, if the lung and serum antiproteinases are not subunits of some primal molecule any relationship between the concentration of one as compared to the other could have some non-genetic, e.g., environmental origin.

Several methods listed below will be employed to study the relationship between antiproteinases of the lung and the serum.

1. Polyacrylamide gel electrophoresis, with and without sodium dodecyl sulfate will be used to determine if the antiproteinases isolated from dog or human lungs have subunits such as easily dissociable or reducable polypeptide chains and if they are similar to serum antiproteinases isolated in a similar chromatography procedure.

2. Antisera will be raised in rabbits injected with purified serum antiproteinase and tested against lung antiproteinase using the immunoelectrophoretic and Ouchterlony techniques to find out whether or not the molecules share antigenic, and therefore, structural sites. As already described in Part II we have prepared antisera to whole dog serum, whole human serum, crude lung antiproteinase and we are presently preparing antisera against purified dog α_1-antitrypsin, α_2-macroglobulin, partially purified lung antiproteinase, as well as against purified human α_1-antitrypsin and human α_2-macroglobulins. The latter two substances were obtained through the generous help of the American Red Cross. We have already established that the partially purified lung antiproteinase contains a component which reacts with antiserum developed against whole dog serum: that this component migrates in immunoelectrophoresis as an inter-alpha protein and that there are no other cross-reactive proteins present in the partially purified preparation. To determine if the cross-reactive protein is the lung inhibitor will require complete purification by procedures described in earlier sections. We will also carry out this immunologic analysis on the human lung lavage in order to bring this work closer to the human emphysema system.

3. Samples of each inhibitor will also be hydrolyzed and subjected to two-dimensional electrophoresis and chromatography, producing two-dimensional peptide maps in order to further compare the primary structure of the proteins.

4. Each inhibitor will be tested against such standard proteolytic enzymes as trypsin, collagenase, papain and elastase using hemoglobin and other pure proteins as substrates to obtain a general idea of its inhibitory spectrum.

5. Each inhibitor will also be tested for its effect on enzymes derived from dog or human polymorphonuclear leukocytes and macrophages, using as substrates material obtained from fractionated lung tissue to more closely approximate its actual effect in the intact animal. These experiments will expand the work already described in Tables I and II.

All these studies will aid us in understanding the nature of the dog and human lung antiproteinases, their interaction with various cellular

proteinases which may be present in the lung under certain physiological conditions and their relationships to dog and human serum antiproteinases. This work would have potential importance in understanding the etiology of emphysema, may aid in making earlier and more accurate diagnosis and may help to develop new techniques of treatment including the possibility of immunotherapy.

The people involved in the studies described above include Drs. George Weinbaum, Philip Kimbel and Bruce Sloan, as well as our biochemistry and histology technicians. The provisional timetable for accomplishing the work described above is approximately 24-30 months. Some of this work will run simultaneously with that described below.

2. The Role of Cigarette Smoke in Determining Proteinase:Anti-Proteinase Interaction

Background

The response of lung tissue to both acute and chronic treatments with cigarette smoke have been amply described micro- and macroscopically. Several laboratories (9,13) have supplied data detailing the numerous changes resulting from in vivo exposure of pulmonary tissue to noxious fumes but none has sought to determine the effect of such exposures on the interaction between autogenous proteinases and antiproteinases.

a. Effect of Smoking on Proteinases from Lung Macrophages, Lung Tissue, and Polymorphonuclear Leukocytes.

Populations of lung macrophages will be obtained by lavage (3). PMNs will be obtained from whole blood by the usual procedures used in our laboratory (23). Lung cells will be prepared by mincing lung tissue and homogenizing in a Potter-Elvehjem homogenizer, disrupted by sonication, and made into acetone powders. The powder will be extracted using water, 1M NaCl, and 8M urea to affect an initial fractionation corresponding roughly to acid, neutral, and alkaline proteinases. Work currently in progress in our laboratory using the PMN material from dog blood (as described in Part II) indicates: 1) the water extract is much richer in acidic proteinase (catheptic) activity than the combined neutral and alkaline activities; 2) the acidic activity is much less in the sodium chloride extract and essentially zero in the urea extract; 3) the sodium chloride extract has the greatest amount of alkaline and neutral activities; 4) the neutral and slightly acidic (pH 5) activities of the urea extract are more significant than either the acidic or alkaline proteolytic activities; 5) elastolytic activity, using t-Boc-L-alanine ester as substrate, is significant only in the high salt and urea extracts; 6) collagenase activity, using bovine achilles tendon as substrate was found in both the water and the low salt extracts, with minimal activity in higher salt or urea extracts. These findings are important in that they 1) demonstrate an initial significant separation of the major proteolytic activities and 2) the two principal enzymatic activities involved in connective tissue destruction, i.e., elastase and collagenase, and implicated in causing disease in these tissues (24) are easily separated. Although routine elastase assays are performed using a synthetic substrate, the use of elastin-orcein has confirmed the fact that the high salt and urea fractions did indeed have the elastolytic activities. As described in

Part II we have succeeded in fractionating the dog PMN further to give us a partially purified enzyme fraction which contains less than 1% of the total cell protein and this fraction is the only one capable of inducing emphysema in isolated dog lobes. This work is being expanded to examine the purification of the emphysema-inducing agent from dog alveolar macrophages, human PMN and human alveolar macrophages utilizing, initially, the same purification scheme which has worked for the dog PMN enzyme.

The emphysema-inducing enzyme from dog PMN has been enriched in lung-digesting capacity. Therefore, those enzymes capable of destroying lung-derived substrates will be employed in the studies dealing with proteinase: antiproteinase interactions.

Macrophages will be washed from lungs of dogs exposed to cigarette smoke. The smoke exposure will be performed using a mask system as described in the letter submitted to Dr. F. Nordsiek on February 8, 1973, or we will use an updated system similar to that described by Battista and co-workers (25). We will rely on the experts recommended by the Tobacco Research Council to select the most satisfactory exposure system. Acute treatment will consist of short exposure to cigarette smoke adjusted to give an amount equivalent to two packs for a smoker of average weight. Chronic treatment will involve using cigarette smoke adjusted to give an amount equivalent to one-half pack in one group and two packs in a second group for varying intervals of time over a 0-6 month period.

The proteolytic activity of the macrophages will be determined using standard protein and synthetic substrates and also the specific lung substrates described above. Proteolytic activity will be measured not only using acetone powders of sonicated cells, but also from supernatants of cells allowed to merely discharge their enzymes due to possible changes in intracellular stability. These determinations of proteolytic activity will be performed on cells from both normal and treated dogs since there are reports (26) that smoking causes an increase in pulmonary proteolytic activity. Such data should be important in ascertaining if certain pollutants can potentiate the development of emphysema via the mechanism of proteolytic degradation.

b. <u>The Effect of Smoking on Serum and Lung Anti-proteinase Levels and Activities</u>.

Sera from dogs subjected to acute and chronic smoking schedules will be assayed for changes of antiproteolytic activities using both the antitryptic assay of Eriksson (27) and the more specific enz/me-substrate systems described above. Inhibition of specific antiproteolytic activities will be followed using the agar gel electrophoretic technique of Ouchterlony (28), in order to demonstrate not only different levels, but also different inhibiting spectra of antiproteolytic agents. The immunoelectrophoretic technique will tell us if there has been a shift in the amounts of α_1-antitrypsin, α_2-macroglobulins and inter-alpha anti-proteinase components by utilizing the specific antisera presently being prepared. These data will, therefore, aid in determining if cigarette smoke affects the level or composition of the various serum anti-proteinases.

Lung antiproteinase isolated as described in an earlier section will be assayed to determine possible changes due to exposure to cigarette smoke. The total amount of lung antiproteinase, the amount bound in the lung to proteinases and the ratio of these values will be determined to give a quantitative picture of the relationship of smoking to antiproteinase activity. Qualitative differences will be sought for by redetermining the spectra of enzymes inhibited by lung antiproteinase after exposure to cigarette smoke and comparing these to normal values.

c. Effect of Cigarette Smoke on Induction of Proteinase-Induced Emphysema.

Emphysema induction will be studied both in vivo and in vitro. Initially, our studies using PMN homogenates to produce emphysema will be extended to dogs previously subjected to various regimens of smoking as described in the preceding section. In our acute in vivo studies dogs exposed to cigarette smoke will be treated with various amounts and types of leukocyte-derived proteinase, sacrificed, and the severity of emphysematous lesions compared to those of untreated dogs. Chronic studies, as described previously, will also be performed prior to proteinase treatment to measure the short and long term effects of exposure to smoke on the ease of emphysema-induction using leukocyte proteinases.

In vitro studies will be carried out by removing lungs from dogs exposed to cigarette smoke and the isolated lobes used as test organs for proteinase studies. This method is not only more economical than whole animal studies but also emphasizes the effects of smoke and proteinases on the lungs themselves, with a minimum of extrapulmonary involvement due to circulatory transport of serum factors to act as antibodies, serum antitrypsin proteins or serum proteolytic enzymes. Previous work in the laboratory has demonstrated the feasibility of this technique for quantitating the amount of enzyme required to induce emphysema in the intact animal and should be of considerable utility in the program.

The significance of this program will lie in its determination of the role played by smoking on the development of emphysema. By studying the effect of cigarette smoke on both the serum and lung antiproteinases we hope to demonstrate which antiproteinase is more important in conditions likely to cause emphysema. We will also better understand the role of proteinase-antiproteinase balance during induction of experimental emphysema. We hope to utilize the observations made in our model system in understanding the sequence of events occurring during the development of human pulmonary emphysema. In accomplishing this we believe that we shall be better able to describe those individuals most prone to emphysema development and, eventually, suggest a method of treatment. The smoking experiments described in this section will only be performed on dog materials. The people involved are the same as listed in the previous specific aim (pg.10). This work will be initiated 6-12 months after characterization of lung antiproteinases has been begun and our suggested timetable is that this work will take 24-30 months to accomplish.

C. REFERENCES

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10. SPACE AND FACILITIES AVAILABLE:

The facilities available in the Biochemistry Laboratory for use during these investigations include:

1. Zeiss phase microscope
2. Sorvall centrifuge
3. Hitachi-Perkin Elmer spectrophotometer
4. Mettler balance
5. Refrigerator and freezer
6. Glassware for all the basic techniques described

The facilities available in the Cardiopulmonary Laboratories for use during these investigations include:

1. Eight-channel FM magnetic tape recorder with voice input.
2. Six-channel FM magnetic tape recorder with voice input.
3. Filtering and differentiator circuit.
4. Differential and vascular pressure transducers.
5. Animal body plethysmograph (modified small body respirator).
6. Spirometers.
7. Gas chromatograph for CO and N_2 analysis.
8. Godart CO Analyzer.
9. Godart CO_2 Analyzer.
10. Beckman O_2 Analyzer.
11. Blood gas and pH electrodes system with water bath and tonometer.
12. Pressure cycled respirator (Bird Mark VII).

The Research Laboratories have the following general facilities available:

1. Hotpack walk-in incubator
2. Walk-in cold room
3. RCA electron microscope
4. Dark room facilities

Animal boarding facilities occupy an adjoining building. An animal surgical suite is located there, contains a completely equipped operating facility and is maintained by a full-time staff. Standardization of dogs is practiced and during a three-week period of observation, testing and treatment prior to experimentation, pre-existing medical problems are eliminated.

11. ADDITIONAL FACILITIES REQUIRED:

NONE

12. BIOGRAPHICAL SKETCHES

A. GEORGE WEINBAUM, Ph.D.

Bioscientist, Pulmonary Disease Section
Born: REDACTED

Role in Project: Co-Principal Investigator

Education, Training, Honors

University of Pennsylvania, Philadelphia, Pa. - A.B.	
The Penna. State University, University Park, Penna. - M.S.	
The Penna. State University, University Park, Penna. - Ph.D.	
Tokyo U. Inst. Appl. Micro, Japan - Postdoctoral	
Albert Einstein Medical Center, Philadelphia, Pa. Post-doctoral	1961
Fulbright Research Scholar, Tokyo University	1959-60
Career Development Awardee, National Inst. of Gen. Med. Sciences	1969-74

Professional Experience

I am presently an Associate Member in the Biochemistry Department. My research at Albert Einstein Medical Center has involved studies on animal and bacterial cell membrane structure and synthesis, enzyme biosynthesis and regulation, biosynthesis of naturally occurring nucleoside analogs in fungi, abnormal cell wall synthesis and characterization of the lipids of E. coli cell wall complexes. I am a Career Development Awardee.

From 1957-61, I was director of the Biochemical Section of the Pathology Department at Geisinger Medical Center, Danville, Penna. My research involved amino acid analogs and tissue culture cells. I spent one year (1959-60) as a Fulbright Research Scholar at the Institute of Applied Microbiology in Tokyo University. I was studying exoenzyme synthesis in B. subtilis.

I received my Ph.D. from Penna. State Univ. in 1957, having worked with Dr. M. F. Mallette on induced enzyme synthesis in E. coli.

B. PAILIP KIMBEL, M.D.

Head, Pulmonary Disease Section
Born: REDACTED

Role in Project: Co-Principal Investigator

Education, Training, Honors

Temple University (Major in Sciences)
Temple University School of Medicine - M.D.
Internship, Albert Einstein Medical Center
Residency, Internal Medicine, Albert Einstein Medical Center
USPHS Post-Doctoral Research Fellowship
Department of Physiology and Pharmacology
Graduate School of Medicine
University of Pennsylvania

Alpha Omega Alpha Honor Medical Society - Award for highest examination scores, senior medical class

Diplomate, American Board of Internal Medicine

Professional Experience

Head, Pulmonary Disease Section, Albert Einstein Medical Center, Philadelphia, Pennsylvania	1961-
Professor of Medicine, Temple University Health Sciences Center School of Medicine, Philadelphia, Pennsylvania	1971-
Associate Member, Research Laboratories, Albert Einstein Medical Center, Philadelphia, Penna.	1968-
Associate Professor of Medicine, Temple University Health Sciences Center School of Medicine	1967-71
Associate in Medicine, Temple University Health Sciences Center School of Medicine	1963-67
Instructor in Medicine, Temple University Health Sciences Center School of Medicine	1960-63
Research Associate, Fels Research Institute, Temple University School of Medicine and Institute for Cancer Research (with Dr. S. Weinhouse - Blood Glucose Metabolism). Part-time	1958-61
Research Associate, Department of Physiology, Graduate School of Medicine, University of Pennsylvania (part-time). Worked with Dr. A.B. Dubois and Dr. H. Linderholm in studies of Pulmonery Capillaty Blood Flow Simultaneously with Cardiac Catheterization	1958-61

REDACTED

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C. BRUCE SLOAN, Ph.D.

Research Associate
Born: REDACTED

Role in Project: Research Associate

Education, Training, Honors

Temple University - B.A.
Hahnemann Medical College - M.S.
Hahnemann Medical College - Ph.D.
Department of Pathology, Harvard Medical School
Post-doctoral
Department of Microbiology, Albert Einstein Medical
Center - Post-doctoral
Temple University - Dean's list
Hahnemann Medical College, U.S.P.H.S. Predoctoral
Fellow

REDACTED

Professional Experience

I am presently a postdoctoral fellow in the Biochemistry Department, Pulmonary Disease Section, at Albert Einstein Medical Center. My research here has consisted of studies on proteolytic enzymes derived from dog polymorphonuclear leukocytes and macrophages. I have been attempting to assay, isolate and define the role of enzymes on the development of experimental emphysema using the dog as a model system.

From 1969-1971, I was a postdoctoral fellow in the Laboratory of Chemical Pathology, Department of Pathology, Harvard Medical School. My research was concerned with the role of genetics and the state of the antigen on cellular and humoral immune mechanisms.

I received my graduate training at Hahnemann Medical College, under Dr. Peter Stelos. My research was concerned with studies of the structure of immunoglobulin G, specific antibodies, and Bence-Jones proteins and employed enzymatic and chemical procedures for protein degradation and sequencing.

13. PUBLICATIONS:

A. G. Weinbaum, Ph.D.

Okuda, S. and Weinbaum, G., Immunologic Cross-Reactivity of Escherichia coli B, Envelope Glycoproteins with Some Animal and Plant Cell Membrane Proteins, J. Immunol., 103:869 (1969).

Weinbaum, G., Fischman, D.A. and Okuda, S., Membrane Modifications in Nutritionally Induced Filamentous Escherichia coli B, J. Cell Biol. 45:493 (1970).

Marco, V., Mass, B., Meranze, D.R., Weinbaum, G. and Kimbel, P., Induction of Experimental Emphysema in Dogs Using Leukocyte Homogenates, American Review of Respiratory Disease, 104:595 (1971).

Mass, B., Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema, American Review of Respiratory Disease, 106:384 (1972).

Kimbel, P., Mass, B., Ikeda, T. and Weinbaum, G.: Emphysema in Dogs Induced by Leukocyte Contents, Pulmonary Emphysema and Proteolysis, p.411. Edited by C. Mittman, Academic Press, Inc., New York (1972).

B. P. Kimbel, M.D.

Kaplan, A.S. and Kimbel, P.: Pulmonary Capillary Blood Flow Waves in Subjects with Abnormal Pulmonary Hemodynamics, Journal of Applied Physiology, 28:793 (1970).

Marco, V., Mass, B., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema in Dogs Using Leukocyte Homogenates, American Review of Respiratory Disease, 104:595 (1971).

Mass, B., Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema, American Review of Respiratory Disease, 106:384 (1972).

Kimbel, P., Mass, B., Ikeda, T. and Weinbaum, G.: Emphysema in Dogs Induced by Leukocyte Contents, Pulmonary Emphysema and Proteolysis, p.411. Edited by C. Mittman, Academic Press, Inc., New York (1972).

Marco, V., Meranze, D.R., Yoshida, M. and Kimbel, P.: Papain-induced Experimental Emphysema in the Dog, Journal of Applied Physiology, 33:293 (1972).

C. B. Sloan, Ph.D.

Sloan, B. The Extension of Thin Layer Electrophoresis on Cellulose to the Identification of DNS-amino acids. J. Chromatography 42:426 (1969).

Sloan, B. and Gill, T.J. Genetic and Cellular Factors in the Immune Response. IV. The Effect of Aggregation on Antibody Formation and on Delayed Hypersensitivity in the Inbred ACI and F344 Strains of Rats. Journal of Immunology, 108:26 (1972).

13. PUBLICATIONS (continued)

Sloan, B. and Gill, T.J. Synthetic Polypeptide Metabolism. IV. *In Vivo* and *In Vitro* Degradation of Poly (Glu^{52} Lys^{33} Tyr^{15}) in Highly Responding (ACI) and Poorly Responding (F344) Strains of Rats. Immunochemistry, 9:677 (1972).

14. FIRST YEAR BUDGET:

	% Time	Amount
A. Salaries		
Professional		
George Weinbaum, Ph.D., Co-Principal Investigator	20	
Philip Kimbel, M.D., Co-Principal Investigator	20	
Bruce Sloan, Ph.D., Research Associate	50	
Technical		
Biochemistry Technician	100	
Histology Technician	50	
Fringe Benefits		
Sub-Total for A		
B. Consumable Supplies		
Animals		2,250
Boarding of animals		750
Glassware		500
Chemicals, Drugs, Stains, etc.		1,000
Sub-Total for B		$ 4,500
C. Other Expenses		
Travel to National Meetings		500
Publication costs		250
Sub-Total for C		$ 750
Running Total of A + B + C		$32,750
D. Permanent Equipment		
2 Animal Smoking Masks @ $250 each		500
Sub-Total for D		$ 500
E. Indirect Costs (15% of A + B + C)	-	4,913
TOTAL REQUEST		$38,163

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15. ESTIMATED FUTURE REQUIREMENTS:

	Salaries	Consumable Supplies	Other Expenses	Permanent Equipment	Indirect Costs	Total
Year 2		$4,500	$750	- 0 -	$5,288	$40,538
Year 3		$4,500	$750	- 0 -	$5,588	$42,838

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16. OTHER SOURCES OF FINANCIAL SUPPORT:

CURRENTLY ACTIVE

Title of Project	Source	Amount	Inclusive Dates
Etiology of Experimental Emphysema.	N.I.H. - HE 13787-01	$140,000	5/1/71 to 4/30/74
Structure, Function and Synthesis of Cell Membranes	N.I.H. - K04-GM-07259-04	$ 23,000	7/1/69 to 6/30/74

PENDING OR PLANNED

Title of Project	Source	Amount	Inclusive Dates
Renewal	N.I.H. - HE 13787-04	$150,000	5/1/74 to 4/30/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to:

Mr. Jerome Baron, Vice-President for Fiscal Affairs

Mailing address for checks:

Albert Einstein Medical Center - N.D.
York & Tabor Roads, Phila., Pa. 19141

Principal Investigators:

George Weinbaum, Ph.D.
Philip Kimbel, M.D.

Signatures:

George Weinbaum Date 1/22/74
Philip Kimbel Date 1/22/74

Responsible officer of institution

Mr. Steven Sieverts
General Director

Signature: Steven Sieverts Date 1/25/74

Telephone: 215 DA 9-0700 Ext. 381

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